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PHYSIOLOGY OF CONTINUOUS BONE MARROW CULTURE DERIVED
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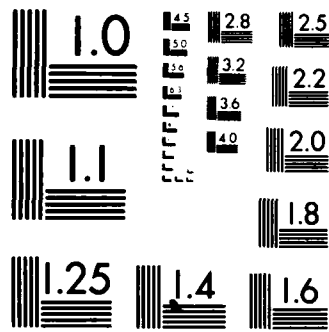
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"PHYSIOLOGY OF CONTINUOUS BONE MARROW CULTURE DERIVED
PERMANENT GRANULOCYTE-MACROPHAGE PROGENITOR CELLS"

FINAL REPORT

Joel S. Greenberger, M.D.

August 31, 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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Summary

During the 24 months of this contract ending August 31, 1984, we have attempted to derive permanent lines of growth factor (Interleukin-3) dependent permanent human hematopoietic progenitor cell lines for transfusion therapy of patients suffering from marrow suppression. This work was to parallel the work we have achieved and published previously with mouse IL-3 dependent hematopoietic progenitor cell lines. The mouse lines derived and reported by us in publications cited in 1982 and 1983 indicated that IL-3 dependent hematopoietic progenitor cell lines of mouse origin would retain the ability to self renew in vitro, producing cumulative cell numbers of over 10^{11} cells over several months passage in a continuous source of IL-3. The cell lines were demonstrated to increase peripheral white blood cell counts in vivo following inoculation by tail vein into sublethally irradiated mice and in several experiments to prolong life following inoculation of the cells in animals that had received sublethal total body irradiation and bacterial infection. To achieve this goal, numerous mouse cell lines were cloned in vitro and permanent lines established and characterized as granulocyte-macrophage in origin by ability to differentiate to cells capable of superoxide production, phagocytosis and killing of bacteria in vitro.

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In our experiments with human continuous bone marrow cultures we attempted to derive permanent nonadherent cell lines from these cultures that would reproduce the mouse results. As a corollary to this, we attempted to derive a permanent source of human Interleukin-3 such that continuous self-renewal of cloned human IL-3 dependent cell lines would be possible. The in vitro correlates we chose to study were those of in vitro phagocytosis and killing of bacteria and in vitro parameters associated with respiratory burst and physiologic parameters of human granulocytes.

The results of the work over these two years have indicated that a source of human IL-3 is clearly a subset of human T-lymphocytes (Greenberger, et al., Experimental Hematology, 1984, in press).(7) We have also demonstrated that the mouse molecularly cloned and expressed gene product for IL-3 has activity stimulating self-renewal of hematopoietic progenitor cell lines and have demonstrated that these cell lines are multipotential and that cells induced to form colonies by the molecularly cloned and expressed IL-3 are multipotential hematopoietic stem cells (Greenberger, et al., Experimental Hematology, 1984B in press).(11) Our work over the past year has been an attempt to derive a permanent source of the cDNA for human IL-3 such that introduction of this by transfection into Cos monkey cells or bacteria vector would enable us to derive a large source of IL-3. The progress in this area has been promising and, with the completion of work on this contract, we will propose to continue this work with

other funding sources as they become available in our laboratory, although the work will of necessity be slowed with the absence of renewal of the present contract.

Specific Accomplishments to Date

We have demonstrated that human HLA-DR positive T-lymphocytes of an inducer subset produce human Interleukin-3. Supernatant from cloned T-cell lines was collected following growth of the cells in serum-free medium and this medium uses a source of IL-3 demonstrating the induction of multipotential hematopoietic cell colonies from fresh human marrow or nonadherent cells derived from human continuous bone marrow cultures.(7) Attempts to establish self-renewing lines of human cloned hematopoietic progenitor cells were unsuccessful using either fresh human marrow, nonadherent cells from human long term marrow cultures, or enriched populations of granulocyte-macrophage progenitor cells derived from either of these sources. The failure to attain such human lines may be attributable to the following:

1. The mouse cell lines were in fact a form of malignant transformation which, although not demonstrating an abnormal karyotype, may have in fact begun to transform.(1,3)
2. The concentration of target cells that are capable of establishing permanent self-renewing lines in human IL-3 may have been too sparse in either source of marrow chosen.(5,6,10,12)

3. The conditions for cloning the cells of human origin were not appropriate and would have to be modified from the method that had been successful with the mouse.

These studies are in progress.

The Establishment of a Permanent Source of the Gene Product of Human IL-3

Our studies with murine IL-3 that had been molecularly cloned and expressed in Cos cells, in collaboration with Drs. Gary Nabel and Harvey Cantor at the Dana-Farber Cancer Institute and the DNAX Corporation in Palo Alto, CA, led to our demonstration that mouse IL-3 is a multipotential CSF capable of inducing self-renewal of murine hematopoietic progenitor cell lines.(11) The cell lines included that line that protected sublethally bacterially infected mice from death.

The mouse molecularly cloned and expressed gene product was found to contain the properties of several biologic activities previously ascribed to several molecules. This data unequivocally demonstrated that a single molecule had multiple biologic activities and confirmed many of our previous publications.

With a cloned cell line of human T-cells that produces an identical molecule, it was now possible to derive a permanent source of this cDNA for human IL-3. This work is

currently in progress and will continue, as funding sources are available.

Human Long Term Marrow Cultures

In the last year, we have begun studies on the molecular biology of the gene for human multipotential CSF (Interleukin-3). We have previously reported in a manuscript submitted for publication, Greenberger, Krensky, Burakoff, Messner, Sakakeeny, Exp Hematol (submitted), that human IL-2 cloned lymphocyte lines with OKT4, OKT8 positivity and T-cell phenotype produce a factor that stimulates multipotential colony formation by human bone marrow cells in vitro. The supernatant from these T-cells induced CFU-GEMM (colonies containing erythroid, neutrophil-granulocyte, macrophage and megakaryocytic cells), as well as colonies composed of pure megakaryocyte (CFU-mega), pure erythroid (BFUe) and large numbers of granulocyte-progenitor cell colonies (GM-CFUc).

We have now begun the molecular biology for molecular cloning and isolation of the gene for human IL-3 with attempts to design a method for expression of this in Cos monkey cells and ultimately in bacteria or yeast fermentation units for large-scale production of the molecule.

We have also begun experiments testing continuous intravenous infusion of murine Interleukin-3, which has been generously provided in collaboration by three investigators. We have obtained purified Interleukin-3 that has been purified to homogeneity from the conditioned medium of WEHI-3 cells by

Dr. James N. Ihle (Frederick Cancer Research Center, Frederick, MD) and have obtained the molecularly-cloned and expressed gene for IL-3 of murine origin from Drs. Gary Nabel and Harvey Cantor at the Dana-Farber Cancer Institute, through their collaoration with the DNAX Corporation in Palo Alto, CA. Data on the biologic activity of purified IL-3 and Cos-MCGF (the molecularly cloned and expressed gene product from the DNAX Corporation) has been provided in the draft of the manuscript currently being prepared and in our abstract to be presented at the American Society for Experimental Hematology (ISEH) in Atlanta, GA, August 1984 (abstract enclosed). We have demonstrated that the molecularly cloned and expressed gene product for murine IL-3 is biologically indistinguishable from the purified IL-3 obtained from the murine cell line WEHI-3 that produces the gene product.

We have begun studies to evaluate the biologic activity of human IL-3 in replating assays in collaboration with Dr. Makio Ogawa and are doing similar experiments in our own laboratory.

The focus of the past several months of the contract proposal has been on the molecular biology of the human IL-3 gene. The methods for this attack are described below.

Molecular Cloning and Expression of the Gene for Human Interleukin-3

We have demonstrated that murine Interleukin-3 stimulates unlimited self-renewal of mouse multipotential

hematopoietic progenitor cells that have potential use in transfusion therapy. We have demonstrated that the cell lines maintained in vitro for prolonged periods in mouse IL-3 maintain a "normal" karyotype, do not produce transformed sublines for over 2-1/2 years and retain biologic functioning in vivo. Thus, the logic for isolation of human IL-3 dependent hematopoietic cell lines for potential transfusion therapy as an alternative to granulocyte support. For this goal, we must first isolate a stable source of production of unlimited quantities of human IL-3.

The past year and a half of research on this contract proposal has been focused on isolation of a source of human IL-3. We have presented data in past quarterly and yearly reports examining T-T-cell hybridomas, human T-cell tumor lines including those transformed by HTLV-I and HTLV-II, provided generously for our testing by Dr. Robert C. Gallo, National Cancer Institute and Dr. David Golde (UCLA School of Medicine). We have also collaborated with Dr. Alan Krensky and Steven Burakoff on tests of supernatant from human IL-2 dependent T-cell lines. As shown in the enclosed manuscript submitted to Experimental Hematology, IL-2 dependent T-cell line F1 and many other clones demonstrated high degrees of release of human multipotential CSF. We therefore have elected to establish a T-cell clone from our own laboratory derived entirely from sources in our laboratory and to prepare a cDNA library from the RNA extracted from this cell line after it has been stimulated with HLA-DR nonidentical target cells.

We have begun the preparation of human IL-2 dependent T-cell clones by expansion of CONA-stimulated human peripheral blood T-cells with a preparation of Interleukin-2 that is obtained from the platelet sludge reservoir of donor blood from our blood bank. This conditioned medium contains high levels of IL-2 and following ammonium sulfate precipitation and dialysis provides a stable source of IL-2 for the growth of human T-cell clones. Human T-cell clones are prepared as described in our manuscript and then a single clone will be expanded to 2×10^8 cells. At the present time, we have approximately 6×10^7 cells from one of these pools of T-cells and anticipate that by April or early May we will have the required 2×10^8 cells to make our RNA extraction for preparation of a cDNA library. The methods for establishment of the cDNA library are identical to those previously published and will be carried out in our laboratory. The RNA extraction is expected to take place in early May 1984 and the preparation of the cDNA library during the months of May and June 1984. We will then begin testing the expression of cDNA clones by translation in frog oocytes using as a target cell population nonadherent cells from our human long-term bone marrow cultures, testing for GM-CFUc formation. Positive cDNA isolates that stimulate GM-CFUc formation will then be tested in the more cumbersome assay for CFU-GEMM. Those pools of cDNA that are positive will then be used to generate more specific clones. The methods will involve extraction of pools of RNA from our T-lymphocyte clone designated TED-1, and

translation of this RNA into oocytes. Postive RNA pools will then be reversed transcribed with reverse transcriptase, generating the cDNA library and these cDNA fragments then molecularly inserted into plasmid vector according to published methods (Yung, Hapel, et al., Nature 307:233-237, January 1984) and these cDNA clones then tested by transfection into Cos monkey cells. Supernatant that expresses the GM-CFUc inducing activity in human marrow cells will then be retested in the CFU-GEMM assay. A positive plasmid vector carrying cDNA from a pool of RNA isolated from our human T-cell line that induces CFU-GEMM will then be considered that containing human IL-3.

It is anticipated that the methodology for amplification of the gene product will be available from several sources within the next six months. Current difficulties with the maximal expression of human IL-2, as currently being produced in Japan, Genetics Institute in Boston, and other companies, have been associated with the difficulty in expression of the gene in large enough quantities in bacteria or yeast. Cos-cell expression is transient and the pools of material that are obtained are enough for laboratory research but not enough for expansion of large clones of human cell lines for potential transfusion therapy, cryopreservation, or study of physiologic activity following large scale transfusions. We therefore anticipate that a long range goal of our contract research will be to establish better methods for expression of our human cDNA for human IL-3 in a suitable fermentation system for production of large amounts of the factor.

Physiology of Human Cell Lines Growing in Human IL-3

We have elected to postpone as a preliminary goal for this 3-month period the analysis of human IL-3 dependent granulocyte clones, since large numbers of these have been frozen down and are available for study later, and our primary goal is to obtain the human IL-3 gene from our human T-cell cloned line. The physiology of human granulocyte-macrophage progenitor cell lines growing in human IL-3 will be studied according to the protocol outlined in our initial contract proposal 1-1/2 years ago. We will be testing cell lines grown in initially Cos-cell expressed human IL-3 for markers associated with granulocyte-macrophage, mast cell, eosinophil, or multipotential stem cell properties in vitro. Those clonal lines that demonstrate granulocyte physiology will then be tested for bacterial killing in vitro and for properties of functioning phagocytic cells in diffusion chambers in irradiated mice. We will then begin preliminary studies on analysis of the effects of these cells in co-culture with human bone marrow, human peripheral blood, and human tumor cells. We will determine whether human cell lines growing in IL-3 from our cloned gene product are capable of extensive self renewal in vitro, capable of differentiating after prolonged passage in suspension culture, and will analyze these for karyotypic variations, possible quiescent harboring of cytomegalovirus, HTLV type I or type II (in collaboration with Dr. Robert C. Gallo of the National Cancer Institute) and

for potential activation of Epstein-Barr virus. We will also be concerned with the interaction of these cells with other populations in co-culture to determine whether there is induction of malignant transformation of allogeneic human T-cells with co-culture with human IL-3 dependent cell lines.

The results of the research in the last three months have been particularly exciting with our clear discovery of a stable source of the RNA for human IL-3. With molecular biology techniques that are available to us, it should be possible to have the gene for human IL-3 in a form that can be expressed in Cos cells for in vitro studies and ultimately for expression in bacteria or yeast for production of large quantities to fulfill our goal of production of 10^{10} - 10^{11} granulocytes from a single bone marrow or peripheral blood harvest.

Chronologic List of All Publications
Supported by This Contract

1. Greenberger JS, Sakakeeny MA, Davis LM, Moloney WC and Reid D. Biologic Properties of Factor-Independent Nonadherent Hematopoietic and Adherent Preadipocyte Cell Lines Derived from Continuous Bone Marrow Cultures. Leukemia Res. (in press)
2. Greenberger JS, Klassen V, Kase K, and Sakakeeny MA. Effects of low dose rate irradiation on plateau phase bone marrow stromal cells in vitro: Demonstration of a new form of non-lethal physiologic alteration of support of hematopoietic stem cells. Int J Rad Oncol Biol Phys (in press)
3. Greenberger JS, Sakakeeny MA, Krensky A, Burakoff S, Reid D, Novak T, Boggs S, and Davisson M. Experimental evidence for the potential usefulness of permanent IL-3 dependent multipotential hematopoietic progenitor cell lines in transfusion granulocyte support therapy: Need for expansion of early passage cells. Proc of the Symposium on Long Term Bone Marrow Cultures, Kroc Foundation, September 1983, Alan R. Liss, New York (in press)
4. Greenberger JS, Cohen GI, and Sakakeeny MA. The development of a system for study of bone marrow transplantation in vitro: Effects of x-irradiation dose rate and chemotherapeutic agents on the isolated bone marrow microenvironment. Proceedings of the Symposium on Long Term Bone Marrow Culture, Kroc Foundation, September 1983, Alan R. Liss, New York (in press)
5. Greenberger JS. Recent modifications and technical improvements in human long term bone marrow cultures. Proceedings of the Symposium on Long Term Bone Marrow Culture, Kroc Foundation, Sept. 1983; Alan R. Liss, New York (in press)
6. DeFabritiis P, Bregni M, Lipton J, Greenberger J, Nadler L, Rothstein L, Korbliing M, Ritz J, and Bast RC, Jr. Elimination of clonogenic Burkitt's lymphoma cells from human bone marrow using 4-hydroperoxycyclophosphamide (4-HC) in combination with monoclonal antibodies and complement, Blood (submitted)
7. Greenberger JS, Krensky AM, Messner H, Burakoff SJ, Wandl U, and Sakakeeny MA. Production of colony-stimulating factor(s) for granulocyte-macrophage and multipotential (Granulocyte/Erythroid/Megakaryocyte/Macrophage) (CFU-GEMM) hematopoietic progenitor cells by clonal lines of human IL-2 dependent T-lymphocytes, Exp Hematol (in press)

8. Kluge NK, Ostertag W, Fusco A, Pennie S and Greenberger JS. Multipotential hemopoietic cell lines isolated from stem cell cultures infected with Friend virus complex (MuLV + F-SFFV) show presence of MuLV but not F-SFFV. J Cell Physiol (submitted)
9. Key L, Carnes D, Weichselbaum R, Greenberger J, and Anast C. Factors made by cells in response to calcitriol exposure are capable of augmenting bone degradation by monocyte monolayers. J Clin Invest (submitted)
10. Greenberger SJ, Rothstein L, DeFabritiis P, Bregni M, Bast RC, Jr., Ritz J, Nadler LM, Lipton JM and Sakakeeny MA. Effects of monoclonal antibody and complement treatment of human marrow on hematopoiesis in continuous bone marrow culture. Cancer Res (in press)
11. Greenberger JS, Humphries RK, Messner H, Ihle JN, Sakakeeny MA. Molecularly cloned and expressed murine T-cell gene product is Interleukin-3. Exp Hematol (in press)
12. Rothstein L, Pierce JH, Aaronson SA, and Greenberger JS. Amphotropic murine retrovirus vector transfer of functioning genetic sequences into human hematopoietic and stromal cells in continuous bone marrow culture. Blood (submitted)

- 1A. Key L, Weichselbaum RR, Rothstein L, Carnes D, Anast C and Greenberger JS. 1,25(OH)₂ Vitamin D₃ stimulates human marrow to produce a factor which increases in vitro bone resorption, ASTR 1983, Los Angeles.
- 2A. Greenberger JS, Key L, Carnes D, Anast C, Weichselbaum R, and Rothstein L. 1,25(OH)₂ D₃ induces adipocyte differentiation and production of a bone resorbing factor by human bone marrow stromal cells in continuous marrow culture. AFCR Eastern, Clin Res 32:686A, 1983.
- 3A. Cassady RS, Daugherty C, and Greenberger JS. Spontaneous morphologic differentiation of Interleukin-3 dependent hematopoietic progenitor cell lines is greater with multipotential compared to unipotential lines. AFCR Eastern, Clin Res 31:685A, 1983.
- 4A. Greenberger JS, Klassen V, Reid D, Kase K, and Sakakeeny MA. Low dose rate irradiation stabilized bone marrow stromal cell synthesis of GM-CSF, and a distinct factor required for formation of multipotential stem cell colonies. ASH, 1983, Blood 62:Suppl.#1, p. 135a, 1983.
- 5A. Greenberger JS, Key L, Carnes D, Anast C, Weichselbaum R, and Rothstein L. 1,25(OH)₂ vitamin D₃ (D₃) induces dose-dependent adipocyte differentiation and production of a bone resorbing factor (BRF) by a clonal marrow preadipocyte cell line or stromal cells in human or mouse continuous marrow culture. ASH, 1983, Blood 62:Suppl.#1, p. 150a, 1983.
- 6A. Greenberger JS, Messner H, Krensky A, Burakoff SA, and Sakakeeny MA. Production of CSF for human multipotential hematopoietic colony forming cells (CFU-GEMM) (Granulocyte/erythroid/macrophage/megakaryocyte) by cloned IL-2 dependent human T-cell lines. ASH, 1983, Blood 62:Suppl.#1, p. 135a, 1983.
- 7A. Greenberger JS, Klassen V, Reid D, Kase K, and Sakakeeny MA. Low dose rate irradiation physiologic effects on purified bone marrow stromal cells in vitro. Radiat Res, March 1984, Orlando, FL
- 8A. Greenberger JS, Klassen V, Reid D, Kase K and Sakakeeny MA. Low dose rate irradiation alters bone marrow stromal cell physiologic interaction with hematopoietic stem cells in vitro. AACR, May 1984.
- 9A. Pierce JH, Rothstein L, Aaronson SA and Greenberger JS. Effects of infection with amphotropic murine leukemia virus on hematopoiesis in human continuous bone marrow cultures. ASCO, May 1983.

- 10A. Greenberger JS, Klassen V, Donnelly T, Fitzgerald TJ, Naparstek E and Sakakeeny MA. Low dose rate X-irradiation induced decrease in growth factor production by plateau phase cloned bone marrow stromal cells persists after repair of proliferative damage. AFCR Nat. 5/84.
- 11A. Pierce, JH, Rothstein L, Aaronson SA, and Greenberger JS. Amphotropic murine leukemia virus (MuLV) pseudotypes of transforming virus genomes induce Epstein-Barr virus transformed lymphoid lines from human continuous bone marrow cultures. AFCR Nat., 5/84.
- 12A. DeFabritiis P, Bregni M, Greenberger J, Lipton J, Nadler L, Korbling M, Schlossman SF, Ritz J and Bast RC Jr. Elimination of malignant clonogenic Burkitt's lymphoma cells from human bone marrow using 4-hydroperoxycyclophosphamide (4HC) in combination with monoclonal antibodies and complement (C'). AACR, 1984.
- 13A. Bregni M, DeFabritiis P, Raso V, Greenberger J, Lipton J, Nadler L, Schlossman SF, Ritz J and Bast RC Jr. Elimination of clonogenic Burkitt's lymphoma cells from human bone marrow using a combination of monoclonal immunotoxins reactive with different tumor cell surface determinants. AARC, 1984.
- 14A. Antman K, Amato D, Lerner H, Suit H, Corson J, Wood W, Proppe K, Harmon D, Carey R, Greenberger J, and Wilson R. Pooled results of two randomized trials of adjuvant (ADJ) adriamycin (ADR) for sarcomas (S): Lack of apparent difference between treatment groups. 4th International Conference on the Adjuvant Therapy of Cancer, 1984.
- 15A. Newburger PE, Speier C, and Greenberger JS. Long term bone marrow culture in Chediak-Higashi syndrome (CHS). Society for Rediatic Research, 1984.
- 16A. Greenberger JS, Pierce JH, Rothstein L and Aaronson SA. Amphotropic murine leukemia virus (MuLV) pseudotypes of transforming virus genomes demonstrate high efficiency infection of stromal and hematopoietic stem cells in human continuous bone marrow cultures. ISEH, Atlanta, Georgia, 8/84
- 17A. Greenberger JS, Naparstek E, Donnelly T, Klassen V, and Kase K. Demonstration of a new form of x-irradiation damage in bone marrow stromal cells that is expressed as physiologic alteration of support for hematopoietic stem cells. ASTRO, Oct. 1984, Washington, D.C.
- 18A. Greenberger JS, Sakakeeny MA, Naparstek E, Ihle J, Humphries KC, Messner H and Reid DM. Comparison of hematopoietic effects of homogeneously purified murine interleukin-3 with a molecularly cloned gene product from inducer T-cells. ISEH, Atlanta, Georgia, 8/84.

19A. Fitzgerald T, Greenberger J and Sakakeeny MA. Radiosensitivity of some human leukemia cells can vary with dose rate. ISEH, Atlanta, Georgia, 8/84.

20A. Fitzgerald TJ, Rothstein L, and Greenberger JS. Effect of x-ray dose rate on the clonagenic survival of normal human granulocyte-macrophage hematopoietic progenitor cells (GM-CFUc) in vitro. ISEH, Atlanta, Georgia, 8/84.

21A. Naparstek E and Greenberger JS. A method for study of hemopoietic effects of continuous intravenous infusion of purified or molecularly cloned and expressed murine lymphokines. ISEH, Atlanta, Georgia, 8/84.

22A. Naparstek E, Donnelly T and Greenberger JS. The effect of x-irradiation and alkylating agents on the production of colony stimulating factor(s) by long term bone marrow cultures. ISEH, Atlanta, Georgia, 8/84.

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